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Severity and Mortality of Experimental Pancreatitis Are Dependent on Interleukin-1 Converting Enzyme (ICE)

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ABSTRACT

Interleukin-1β (IL-1β) is produced in large amounts during acute pancreatitis and is believed to play a role in disease progression. Because secretion of IL-1β is dependent on intracellular processing of pro-IL-1β by IL-1 converting enzyme (ICE), we aimed to determine the efficacy of a novel ICE inactivator (VE-13045) in inhibiting secretion of active IL-1β *in vivo* and if the loss of ICE activity would affect the severity and mortality of experimental pancreatitis. Severe hemorrhagic pancreatitis was induced in adult rats by infusion of bile acid into the pancreatic duct. Animals were randomized to receive VE-13045 or vehicle before induction of pancreatitis. To confirm our findings and to ensure that the results were not model dependent, a second series of experiments was conducted using mice possessing a homozygous knockout of the ICE gene in which lethal pancreatitis was induced by feeding a choline-deficient, ethionine-supplemented diet. The severity of pancreatitis was assessed for both experiments by standard surrogate markers, blind histologic grading, and serum IL-1β and tumor necrosis factor-α (TNF-α) levels. Pancreatic IL-1β mRNA induction was assessed by differential RT-PCR. Acute pancreatitis was associated with a 120-fold increase in IL-1β mRNA, which was not affected by ICE inhibition or gene deletion. Cytokine processing and secretion were affected, as evidenced by decreased serum levels of IL-1β and TNF-α ($p < 0.001$) in all animals with an inactive ICE enzyme. This lack of cytokine production increased survival from 32% to 78% following bile salt pancreatitis ($p < 0.01$) and from 24% to 80% following diet-induced pancreatitis ($p < 0.005$). Both ICE-defective groups demonstrated decreased pancreatic necrosis, edema, inflammation, wet weight (all $p < 0.05$), and amylase and lipase ($p < 0.01$). *In vivo* blockade or genetic deletion of ICE inhibits pancreatitis-induced secretion of proinflammatory cytokines without altering IL-1 mRNA production and is associated with decreased pancreatitis severity and dramatic survival benefits.

INTRODUCTION

The cytokine interleukin-1β (IL-1β) plays a pivotal role in acute inflammatory conditions and recently has been implicated in the pathogenesis of acute pancreatitis, a noninfectious inflammatory condition of the pancreas.[1–3] Concomitant with the elevation of pancreatic enzymes, IL-1β can be found in the serum of humans or experimental animals, with serum cytokine levels peaking concomitant with the severity of pancreatic inflammation.[4–7] Several studies have demonstrated the production of large amounts of IL-1β mRNA in the pancreatic parenchyma within hours of the induction of pancreatitis.[8,9] Soon afterward, IL-1β gene expression is upregulated in specific distant tissues, such as the liver and lung[8–10]—organs that characteristically account for the vast majority of pancreatitis-associated morbidity and mortality.[1,2]

Evidence for the importance of this cytokine in the pathogenesis of pancreatitis has been observed in studies that antagonized circulating IL-1α and IL-1β by administration of a recombinant IL-1 receptor antagonist (IL-1Ra). Prophylactic or therapeutic IL-1 blockade was shown to decrease pancreatic destruction and improve survival.[11–13] Furthermore, when acute pancreatitis is induced in animals possessing a homozygous deletion of the IL-1 type 1 receptor (p80), maximal inflammation and pancreatic destruction could not be instilled.[14]

IL-1β is known to be produced as an inactive 31 kDa precursor that undergoes posttranscriptional modification before being secreted.[3,15–17] This processing is dependent on the cys-

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teine protease IL-1 converting enzyme (ICE), which cleaves IL-1β into its active 17 kDa form. Several irreversible peptidyl inactivators of ICE have been reported recently.[18,19] VE-13045 is one such novel compound that irreversibly and systemically inhibits (inactivates) ICE *in vivo* when administered in the peritoneal cavity. This compound has been shown to decrease lipopolysaccharide (LPS)-induced IL-1α and IL-1β secretion and to block the progression of chronic collagen-induced polyarthritis in mice.[19] This compound is not active in these models when administered orally.

The current study was designed to further delineate the importance of IL-1 in the development of pancreatitis and to investigate the mechanism by which it is produced in the course of this disease. Additionally, the *in vivo* efficacy of the ICE inhibitor VE-13045 in decreasing pancreatitis-associated cytokine release and its effects on the severity and mortality of severe lethal pancreatitis were assessed.

MATERIALS AND METHODS

Animal models

Animal studies were performed at an AAALAC-accredited facility in accordance with the Department of Laboratory Animal Medicine at the University of South Florida. Severe lethal pancreatitis was induced by one of two well-described models.

In the first method, acute hemorrhagic necrotizing pancreatitis was induced by the retrograde infusion of bile acid into the pancreatic duct of rats.[13] Briefly, adult male Sprague-Dawley rats ($n = 24$) (250–300 g) were fasted for 24 h and then anesthetized with sodium pentobarbital (50 mg/kg). The duodenum was exposed through an upper midline incision using aseptic technique. The pancreatic duct was cannulated under 10× magnification with PE-10 tubing transduodenally and tied into place around the proximal biliopancreatic duct. A noncrushing clamp was placed across the hepatoduodenal ligament to prevent the injectate from ascending into the biliary tree and liver. Glycodeoxycholic acid (1 mg/kg of 4% solution in phosphate-buffered saline, PBS) (Sigma, St Louis, MO) was injected into the bile duct over 10 min at a pressure never exceeding 10 cm H_2O using a Harvard pump with an inline pressure transducer. Following the injection, the catheter and clamp were removed, and the abdomen was closed in layers. Sham animals ($n = 8$) underwent identical surgical procedures and duct cannulation without bile acid injection.

Inhibition of the ICE enzyme during bile acid pancreatitis was established by the i.p. administration of VE-13045 (15 mg/kg) (Vertex Pharmaceuticals, Cambridge, MA), a recently described irreversible inhibitor of ICE.[18,19] The antagonist or vehicle was administered to random animals 2 h before and 8 h after pancreatitis induction. The compound was formulated for i.p. administration in olive oil:ethanol:DMSO (90:5:5, v/v/v) as described in Ku et al.[19] The rest of the animals received i.p. injections of vehicle (sterile olive oil) on the same schedule.

The second series of experiments used a model of acute hemorrhagic, necrotizing pancreatitis, which was induced in young female mice (15.4 ± 0.7 g) by feeding of a choline-deficient, ethionine-supplemented (CDE) diet (Harlan Teklad, Madison, WI) for 72 h.[12] Animals were fasted overnight before beginning the diet and were allowed water *ad libitum*. Feeding trays were changed every 6 h to ensure sanitary conditions. Regular chow replaced the experimental diet after 72 h.

Rather than pharmacologic inhibition of ICE, as in the first series of experiments, transgenic C57BL/6 mice possessing a homozygous deletion of the ICE gene ($n = 60$) were used in the CDE model of pancreatitis. Wild-type C57BL/6 animals served as controls ($n = 60$). Transgenic mice with a disrupted ICE gene were provided as a breeding pair as a generous gift from Vertex Pharmaceuticals and the Howard Hughes Medical Institute and were bred in the transgenic facility at the University of South Florida under strict isolation and in compliance with all AAALAC guidelines for the breeding of transgenic animals. Knockout animals used in these experiments have been shown to be homozygous (−/−) for the knockout event by reverse transcription polymerase chain reaction (RT-PCR) of genomic DNA using primers[18] and methods previously described by our laboratory.[9,14] These animals have been well described and have been shown to lack IL-1 production in *in vitro* experiments.[18] The ICE gene disruption does not affect development, growth rate, or fertility of the animals and does not produce overt effects on the immune system.[18]

Pancreatitis severity

The severity of pancreatitis was verified in all animals by blind histologic grading of fixed pancreatic sections (necrosis, vacuolization, edema, and inflammation) as described by our laboratory previously.[11,12] Severity was also assessed by comparisons of serum amylase, lipase, blood urea nitrogen (BUN), creatinine, and calcium, which were determined on an automated Kodak Ectachem 700 analyzer (Kodak, Rochester, NY). All rats had measurements of ascites volume and pancreatic weight/body weight ratios calculated. The CDE model in the mouse does not allow for these measurements.[11,12]

Tissue preparation

Following pentobarbital anesthesia (50 mg/kg i.p.), a minimum of 6 surviving animals from each experimental group were killed during maximal pancreatitis (rats at 24 h, mice at 72 h) by exsanguination via cardiocentesis. These time points are well established for both models of pancreatitis to coincide with maximal pancreatic inflammation and cytokine production.[9–14] Intracardiac injection of sterile PBS was used to purge the circulatory system, and then the pancreas was immediately excised and divided for light microscopy and RNA determination. Total RNA was isolated by guanidium thiocyanate/acid phenol extraction as previously described.[20] The integrity of isolated RNA was verified by equimolar 18S and 28S ribosomal RNA bands following denaturing electrophoresis.

Measurement of tissue cytokine mRNA by quantitative differential RT-PCR

Total cellular RNA was primed using oligo $(dt)_{12\text{-}15}$ (Gibco, Gaithersburg, MD) and then reverse transcribed utilizing SuperScript II reverse transcriptase (Gibco). The prepared cDNA was subjected to differential PCR with rat-specific and

murine-specific primers for IL-1β and β-actin obtained from Stratagene (La Jolla, CA). The sequence for IL-1β was (rat sense = 5′-CAGGATGAGGACATGAGCAC-C3′, and antisense = 5′-CTCTGCAGACTCAAACTCCA-C3′; murine sense = 5′-CAGGATGAGATGAGCACC-3′ and antisense 5′-CTCTGCAGACTCAAACTCCAC-3′). The sequence for β-actin was (rat sense = 5′-GTGGGCCGCTCTAGGCACCA-3′, and antisense 5′-CGGTTGGCCTTAGGGTTCAGGGGGG-3′; and murine sense 5′-GTGGGCCGCTCTAGGCACCA-3′ and antisense 5′-CGGTTGGCCTTAGGGTTCAGGGGGG-3′). The IL-1β cDNA products were coamplified with β-actin for 30 cycles using a UNO-Thermoblock (Biometra, Tampa, FL). The reaction products were subsequently visualized by electrophoresis in 2.5% Metaphor agarose (FMC Bioproducts, Rockland, ME) containing ethidium bromide. Ultraviolet illumination was used to visualize the DNA bands, and the gels were photographed digitally and stored on computer disc. Band intensity was determined by optical density with individual cytokine/β-actin cDNA ratios compared using Sigma Scan software (Jandel Scientific, San Rafael, CA). All primers are known to span at least one intron. The internal standard (β-actin) has been shown previously by our laboratory to be linear and unaffected by the progression of pancreatitis throughout the time course of the experimental models used.[8,9,20] Additionally, β-actin maintains a linear relationship with IL-1β from 20 through 40 amplification cycles.[9,20] Restriction digestion of the resulting cDNA products via Pst1 yielded the anticipated 340 and 103 bp fragments (rat IL = 1β and β-actin, respectively) and 17 and 245 bp fragments (murine IL-1β and β-actin, respectively) (data not shown).

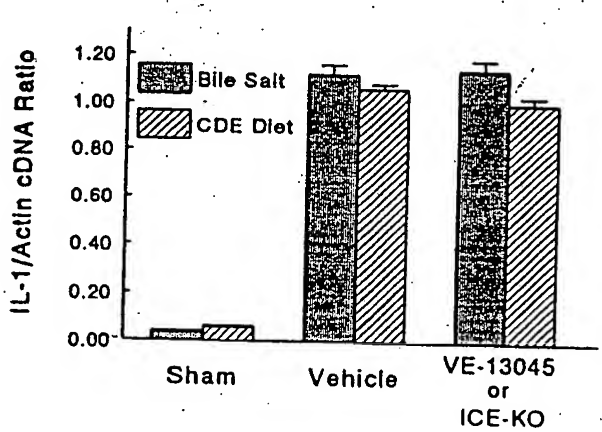

FIG. 1. Intrapancreatic IL-1β gene induction during severe acute pancreatitis. Two models of pancreatitis are shown. Rats underwent bile salt infusion of the pancreatic duct to produce severe hemorrhagic pancreatitis. Mice were fed a choline-deficient, ethionine-supplemented (CDE) diet to produce severe necrotizing pancreatitis. The cDNA ratios of IL-1β and the internal standard β-actin are compared after co-amplification via differential RT-PCR for 30 cycles. Sham animals do not develop pancreatitis and show very low constitutive expression of IL-1β mRNA within pancreatic tissues. The induction of bile salt or CDE diet pancreatitis increases IL-1β mRNA approximately 120-fold ($p < 0.001$), which is not affected by inactivation of ICE by VE-13045 in rats with bile salt pancreatitis or genetic deletion of the ICE gene in mice with CDE diet-induced pancreatitis.

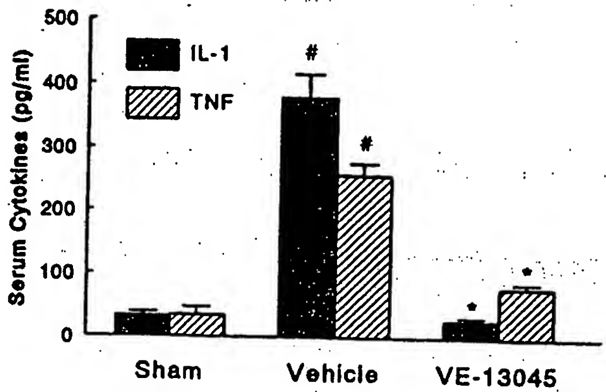

FIG. 2. Serum IL-1β and TNF-α during bile salt-induced acute pancreatitis. Serum was obtained at 24 h after induction of pancreatitis when pancreatic inflammation and cytokine production are known to be at their peak. Severe pancreatitis is associated with significant elevations in serum IL-1β and TNF-α ($p < 0.001$ vs. sham, denoted by #). Inhibition of ICE by VE-13045 eliminates the rise in serum IL-1β while significantly attenuating the rise in TNF-α (both $p < 0.005$ vs. vehicle, denoted by *).

Serum cytokine determination

Serum cytokines were assessed during maximal pancreatitis via murine-specific or rat-specific ELISA as per the manufacturer's direction (Genzyme, Boston, MA). All samples were measured in triplicate by a single investigator (G.K.), who was blinded to treatment group.

Statistical analysis

Results are expressed as means ± SEM. Statistical analysis was performed using the StatS 3 statistical program (Spreadware, Palm Desert, CA) applying the unpaired two-tailed Student's t-test, with significance being assigned to p values < 0.05 unless stated otherwise.

RESULTS

Cytokine production

There was little or no constitutive expression of IL-1β mRNA in the pancreas, although the progression of severe pancreatitis was associated with a near 120-fold increase in the IL-1β/β-actin mRNA ratio ($p < 0.001$ vs. baseline) (Fig. 1). Administration of the ICE inhibitor VE-13045 had no discernable effect on this upregulation in any of the animals examined (p = NS compared with sham). Similarly, those animals expressing a homozygous deletion of the ICE gene demonstrated nearly identical upregulation of IL-1β mRNA in response to the CDE-induced pancreatitis, as did the wild-type animals. Genetic deletion or pharmacologic inactivation of ICE, therefore, had no effect on IL-1β mRNA induction during pancreatitis.

Both IL-1 and tumor necrosis factor (TNF) protein were absent or present in very low concentrations in the serum of normal animals. Acute pancreatitis was associated with a marked elevation of both these cytokines, which was attenuated signif-

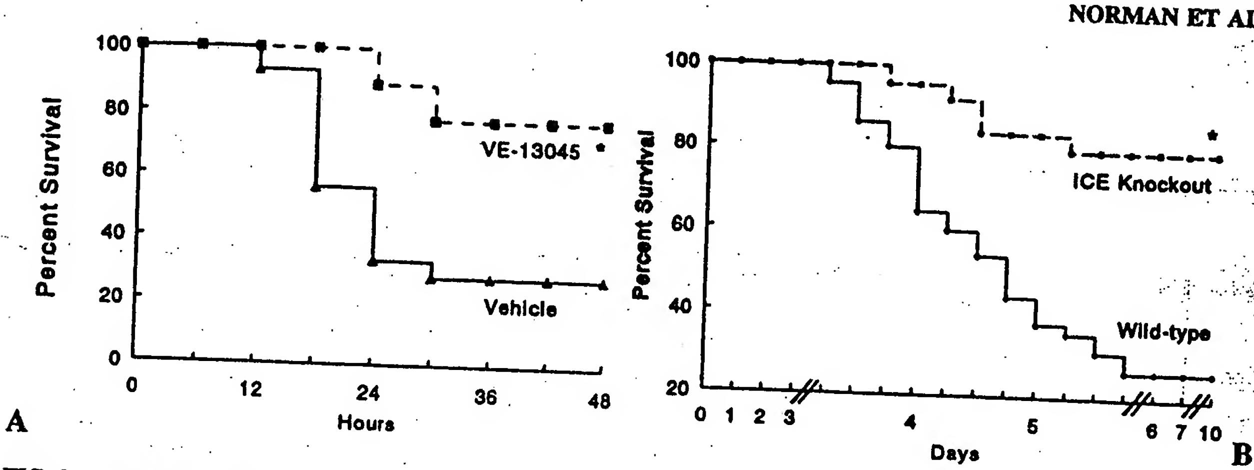

FIG. 3. The effect of ICE inactivation on the mortality of severe pancreatitis. Bile acid-induced pancreatitis ($n = 12$/group) had a mortality rate of 68%, which was decreased to 22% in those animals receiving the ICE inhibitor VE-13045 (A) ($p < 0.01$, denoted by *). The CDE diet-induced model of pancreatitis ($n = 60$/group) had a mortality rate of 73% in wild-type animals, which was decreased to 20% in those animals possessing a homozygous deletion of the ICE gene (B) ($p < 0.005$, denoted by *).

icantly in those animals that received the ICE antagonist (both $p < 0.01$) (Fig. 2).

Mortality

Retrograde infusion of bile acid into the pancreatic duct produced a severe hemorrhagic, necrotizing pancreatitis with a mortality rate of 68%. Inhibition of ICE activity via administration of VE-13045 attenuated the mortality rate to 22% (Fig. 3A) ($p < 0.01$). Similarly, the CDE diet produced a severe necrotizing pancreatitis with a 10-day mortality rate of 73% in normal wild-type mice. Genetic deletion of the ICE gene was associated with only a 20% mortality (Fig. 3B) ($p < 0.005$ vs. wild-type).

Pancreatitis severity

All experimental animals developed pancreatitis. Bile salt-induced pancreatitis was associated with the development of marked pancreatic edema and free ascitic fluid. Those animals receiving the ICE inhibitor showed a significant reduction in pancreatic wet weight ($p < 0.05$) but not ascitic volume ($p = 0.47$) (Fig. 4). Serum amylase, lipase, and BUN were also significantly attenuated with ICE inhibition/deletion (all $p < 0.05$), whereas serum calcium and creatinine were not affected (Table 1). Histologic scoring of pancreatic tissues in animals deficient in the ICE gene, as well as those receiving VE-13045, demonstrated reduced edema, necrosis, and inflammatory cell infiltrate (all $p < 0.05$) but not vacuolization (Table 2).

DISCUSSION

Although several serine proteases are capable of processing the IL-1β precursor to one of several bioactive forms, ICE is the only known protease that generates the mature 17 kDa cytokine with its typical Ala[118] amino-terminus.[3,16–18] The importance of this processing enzyme in regulation of cytokine release has been studied recently *in vitro* using monocytes isolated from mice possessing a homozygous deletion of the ICE gene.[18] These investigators demonstrated that ICE was critical to the cellular export of mature, bioactive IL-1β. Moreover, the inability of monocytes to process IL-1β resulted in substantial reductions in IL-1α, TNF-α, and IL-6 production, demonstrating further the importance of IL-1β to the cytokine cascade in general.

There were two experimental strategies available to ascertain the role of ICE in models of pancreatitis. First, we used an irreversible inhibitor of endogenous ICE to prevent its enzymatic activity. The compound we chose for this purpose (VE-13045) is an esterified form of a highly efficient inactivator of ICE (k_{inact} of 1×10^6 M^{-1} s-1). The esterified form of this compound is a more potent cellular inhibitor of ICE and is biologically active in suppression IL-1β secretion *in vivo*.[19] Although the compound is cleared rapidly from the systemic circulation, its ability to irreversibly inactivate the enzyme *in*

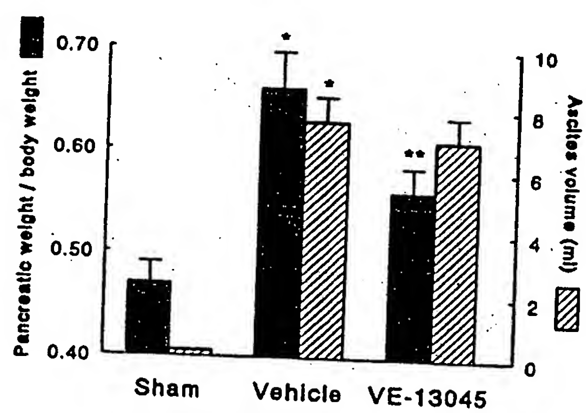

FIG. 4. The effect of ICE inactivation on pancreatic weight and ascites volume in rats with severe pancreatitis induced by bile reflux into the pancreatic duct. The pancreatic weight/body weight ratio increased significantly in vehicle control rats, which developed bile salt pancreatitis ($p < 0.05$ vs. sham, denoted by *). Inactivation of ICE by VE-13045 attenuated this rise by nearly 50% ($p < 0.05$ vs. vehicle, denoted by **). These same animals also developed massive free ascites (vehicle control $p < 0.01$ vs. sham, denoted by *), which was not affected by VE-13045 administration ($p = 0.47$ vs. vehicle control).

TABLE 1. SEVERITY OF PANCREATITIS

	Bile acid pancreatitis			CDE diet pancreatitis		
	Sham	*Vehicle*	*VE-13045*	*Baseline*	*Wild-type*	*Knockout*
Serum amylase (IU, thousands)	0.96 ± 0.7[a]	7.32 ± 0.58*	6.10 ± 0.35*,**	0.54 ± 0.03	28.3 ± 2.6*	15.4 ± 1.2*,**
Serum lipase (IU, thousands)	0.08 ± 0.01	3.76 ± 0.7*	1.46 ± 0.2*,**	0.38 ± 0.04	18.2 ± 1.7*	11.9 ± 0.8*,**
BUN (mg/dl)	14.1 ± 1	91 ± 7*	74 ± 5*,**	15.3 ± 1	46 ± 5.1*	39 ± 3.2*
Calcium (mg/dl)	9.6 ± 0.3	7.7 ± 0.4*	8.7 ± 0.5*	9.4 ± 0.2	9.2 ± 0.5	9.2 ± 0.6
Creatinine (mg/dl)	0.4 ± .01	1.4 ± .02*	1.3 ± 0.01*	0.6 ± 0.01	1.2 ± .08*	1.1 ± .03*

[a]Values are mean ± SEM.
*Significance from sham or baseline values ($p < 0.01$).
**Significance from vehicle or wild type ($p < 0.05$).

vivo when delivered i.p. made it suitable as a probe for the role of ICE in a rapid, acute model of pancreatitis. Use of an exogenous ICE inhibitor allowed us to precisely time the dosing of the compound with respect to disease stimulus.

Our second strategy involved using a transgenic mouse with a disrupted ICE gene. Although the transgenic mice we used show near total suppression in LPS-induced secretion of IL-1α and IL-1β and partial suppression of serum TNF-α and IL-6, they present an alternative to the use of an ICE inactivator that potentially inactivates other cysteine protease homologs of ICE. Use of the knockout mouse, therefore, is the most precise method available for studying the effects of deleting the activity of a single enzyme *in vivo*.

The two models of pancreatitis used in the current study are known to be associated with systemic cytokine production that is independent of endotoxin.[10,21–25] The dramatic rise in IL-1β mRNA within the pancreas and lung has been demonstrated previously and closely mimics pancreatitis progression.[8,10] The current series of experiments demonstrates the efficacy of VE-13045 in antagonizing ICE *in vivo* and confirms the importance of ICE in the processing and secretion of IL-1 as well as affecting total TNF production *in vivo*. The animals with pharmacologic blockade of ICE had cytokine production attenuated to an equivalent degree as the knockout animals, suggesting complete enzyme inactivity. Although the process by which IL-1β is prevented from entering the serum is fairly well characterized, the mechanism by which TNF secretion is attenuated is not known, as it is not a direct substrate of ICE. This decrease in circulating TNF has been demonstrated also in animals pretreated with IL1-Ra before pancreatitis induction[11–13] and is likely due to an overall decrease in the associated systemic inflammatory response and attenuated pancreatitis severity.

The importance of uninhibited production of IL-1β to the overall progression of acute pancreatitis is apparent in these experiments. By preventing the development of the typical cytokine cascade, nearly every measure of pancreatic damage was diminished. Previous experiments in a nonlethal pancreatitis model demonstrated that an active IL-1 receptor is required for the development of maximal necrosis and pancreatic edema.[14] These findings were confirmed in the two lethal models used in this study, but additional benefits were seen, including decreased pancreatic inflammation and less severe changes in serum BUN. It is possible that some of the decrease in pancreatic damage may be attributed to inhibition of ICE-mediated apoptosis, but little can be said about what effect this mechanism has on the other parameters of pancreatitis, as little is known about the role of apoptosis in the development of pan-

TABLE 2. HISTOLOGIC GRADING OF PANCREATITIS[a]

	Bile acid pancreatitis			CDE diet pancreatitis		
	Sham	*Vehicle*	*VE-13045*	*Baseline*	*Wild-type*	*Knockout*
Edema	0	3.3 ± 0.2[b],*	0.9 ± 0.1*,**	0	3.1 ± 0.2*	2.6 ± 0.2*†
Necrosis	0	3.2 ± 0.1*	0.6 ± 0.1*,**	0	3.8 ± 0.1*	3.0 ± 0.1*,**
Inflammation	0	3.1 ± 0.1*	0.7 ± 0.1*,**	0	3.6 ± 0.1*	2.7 ± 0.2*,**
Vacuolization	0	2.8 ± 0.2*	2.6 ± 0.1*	0	2.4 ± 0.2*	2.5 ± 0.1*

[a]Histologic sections were graded in a blinded fashion. Normal tissues were assigned a value of 0, and maximal severity for parameter was assigned a value of 4.
[b]Values are mean ± SEM for 10 fields from each pancreatic specimen from all animals.
*Significance from sham or baseline values ($p < 0.01$).
**Significance from vehicle or wild type ($p < 0.05$).

creatitis. Combined with the dramatic survival benefit attributed to ICE inhibition, these studies demonstrate the profound detrimental effect of IL-1β during acute pancreatitis and suggest that ICE blockade has potential therapeutic applications in this disease.

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